

# **EXHIBIT C**

*Int. J. Cancer*: 66, 130–134 (1996)

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Publication of the International Union Against Cancer  
Publication de l'Union Internationale Contre le Cancer

## N-NITROSODIMETHYLAMINE-DERIVED O<sup>6</sup>-METHYLGUANINE IN DNA OF MONKEY GASTROINTESTINAL AND UROGENITAL ORGANS AND ENHANCEMENT BY ETHANOL

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**N-nitrosodimethylamine (NDMA) is a human cancer initiator suspect. Ethanol, a cancer risk factor, may synergize with nitrosamines by suppressing hepatic clearance, to increase internal exposure. A limitation to these hypotheses is lack of activation of NDMA by many rodent tissues. However, systematic primate studies are lacking. Patas monkeys were utilized to investigate NDMA activation by primate tissues *in vivo*, generating the promutagenic DNA lesion O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG). Adult monkeys received 0.1 mg/kg NDMA by gavage, in some cases preceded by ethanol. Four hours after NDMA only, O<sup>6</sup>-meG was detected in DNA from all tissues. Levels were highest in gastric mucosa and liver and were only about 50% lower in DNA from white blood cells, esophagus, ovary, pancreas, urinary bladder and uterus. With ethanol co-exposure, amounts of O<sup>6</sup>-meG increased at least 2-fold in all tissues except liver. The largest effect was in esophagus (17-fold increase), followed by ovary, large intestine, urinary bladder, spleen and cerebellum (9- to 13-fold increases), and uterus, cerebrum and brain stem (7- to 8-fold increases). Alkylguanine alkyltransferase activities varied over a 30-fold range and were highest in liver and stomach. Thus primate tissues, especially those of the gastrointestinal and urogenital organs, are sensitive targets for DNA adduct damage due to NDMA, and ethanol co-exposure leads to striking increases in adducts. Our data support epidemiology implicating nitrosamines in causation of cancers of stomach and other organs, and alcohol as enhancing internal exposure to nitrosamines.**

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Nitrosamines, which are potent carcinogens in experimental animals and ubiquitous environmental agents, have been long suspected as contributors to human cancer risk (Magee, 1989; Mirvish, 1995). In particular, attention has been given to their participation in causation of smoking-related cancers of upper aerodigestive tract, lung, pancreas, kidney, urinary bladder and large bowel; cancers of the stomach related to dietary nitrosamines and/or intragastric nitrosation; and neoplasms of several of these organs associated with infection by microbes with nitrosating capacity (Magee, 1989; Mirvish, 1995). Epidemiological confirmation of these hypotheses has been difficult, since occurrence of nitrosamines is both widespread and associated with complex mixtures. A role for the most commonly found nitrosamine, *N*-nitrosodimethylamine (NDMA), in most of these cancers has been questioned, because of lack of ability of the corresponding tissues in rodents to activate it to a DNA-damaging form, and absence of tumors in these rodent organs after *in vivo* exposure (Labuc and Archer, 1982; Von Hofe *et al.*, 1987).

However, it has been uncertain whether similar limitations pertain to humans. Human tissues have been shown to metabolize NDMA (Harris, 1987), but the meaning of this with regard to *in vivo* effects has been unclear. We have therefore utilized a non-human primate model, the patas monkey, to determine relative levels of a promutagenic DNA adduct formed *in vivo* by NDMA, O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG). The important contribution of such alkyl DNA adducts to cancer etiology has been evidenced by recent demonstrations of protective effects of overexpression of the enzyme that repairs

them, alkylguanine alkyltransferase (Gerson *et al.*, 1994), and by demonstration in humans of alkylation of DNA in gastrointestinal tissues (Hall *et al.*, 1991) and of association between levels of O<sup>6</sup>-meG in leukocytes and geographical prevalence of gastric cancer (Forman *et al.*, 1994).

NDMA, at low *in vivo* concentrations, is activated primarily by cytochrome P450 2E1 in all species, including humans (Yang *et al.*, 1990). Previously, we observed that patas monkey liver contains P450 2E1 with a molecular size and NDMA demethylase activity similar to those of humans (Anderson, 1992). A pharmacokinetic study indicated that a high percentage of NDMA clearance in patas monkeys could be extrahepatic (Gombar *et al.*, 1990), in contrast to rodents, where metabolism occurs almost exclusively in liver, and with obvious implications regarding potential genotoxic damage in extrahepatic organs.

A related human cancer risk issue is the mechanism of risk enhancement by consumption of alcoholic beverages. Such consumption greatly increases the risk of cancers of the liver and upper aerodigestive tract, and in the case of the latter tissues, further strongly synergizes with tobacco use (Blot, 1992). A number of hypotheses have been put forward to explain this effect of ethanol and/or the congeners in beverages, ranging from solubilization effects, enhancement of cellular entry of carcinogens and inhibition of DNA repair, to tumor promotion (Seitz *et al.*, 1992; Blot, 1992; Mufti, 1992). We and others have noted that, thus far, the largest and most consistent *in vivo* effect of ethanol yet described is enhancement of exposure of internal organs to nitrosamines caused by ethanol-mediated inhibition of first-pass hepatic clearance (Swann *et al.*, 1984; Anderson, 1992; Anderson *et al.*, 1995).

Thus, in mice, ethanol given simultaneously with a single oral dose of NDMA caused 8- to 10-fold increases in the area-under-the-blood-concentration-vs.-time curve (AUC, the best measure of internal exposure), in levels of O<sup>6</sup>-meG in lung DNA, and in number of lung tumors (Anderson, 1992; Anderson *et al.*, 1995). The effect was also seen after chronic dosing in drinking water, was cumulative over a long time course, and eventually involved kidney as well as lung. Absence of an effect on tumorigenesis, when the ethanol and NDMA did not reach the liver simultaneously, confirmed the suppression-of-clearance mechanism.

A similar profound effect of ethanol on clearance of NDMA was also demonstrated in patas monkeys, at blood levels of ethanol comparable to those achieved in humans (Anderson *et al.*, 1995). After an ethanol dose of 1.6 g/kg, clearance of 1 mg/kg NDMA was completely suppressed for at least 6 hr. At approximately the same blood ethanol levels, the increases in AUC for NDMA were similar in monkeys and mice. It was

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therefore of interest to determine whether the increased exposure of the internal organs would result in increased alkylation of DNA in monkeys, as in mice.

#### MATERIAL AND METHODS

Male and female *Erythrocebus patas* monkeys were maintained under conditions approved by the American Association for Accreditation of Laboratory Animal Care; they were 4 to 12 years old and weighed 5–6 kg (females) or 12–13 kg (males). Some of the monkeys used in this study had been exposed to carcinogens, but none within the previous 5 years. The inter-monkey reproducibility of the data confirmed that these earlier treatments did not affect the outcome of the present experiments. The monkeys were trained to accept oral administration of chemicals without anesthesia, by use of physical restraint and treat reward. NDMA (Sigma, St Louis, MO) was administered in sterile water (2 ml/kg), by gavage. Ethanol was given 45 min before the NDMA, 1.6 g/kg (8 ml/kg of a 20% solution), also by gavage.

In Series I, 3 female monkeys were used for blood collection only, and they each were treated in 4 sequential experiments, at 2 week intervals, with 0.1 mg/kg NDMA; this dose plus ethanol; 1 mg/kg NDMA; and the latter NDMA dose plus ethanol. Blood samples were centrifuged at 2,500 rpm for 30 min and the cell fraction frozen.

In Series II, 7 monkeys were treated and killed for collection of tissues, 5 with NDMA (0.1 mg/kg, 3 females, 2 males) or 2 with ethanol/NDMA (2 females). In view of the between-monkey reproducibility of the data, it was decided that 2 monkeys were sufficient for the latter treatment, in keeping with the general policy to utilize as few monkeys as possible. For 2 females of each treatment group, 2 ml of blood was drawn at 20 min, 40 min, and 2 hr. All were killed at 4 hr by treatment with a high dose of ketamine, followed by exsanguination; organs were removed as rapidly as possible and placed on wet ice for dissection and separation of mucosal surfaces, and tissue pieces were frozen at  $-80^{\circ}\text{C}$ . Assays for content of  $\text{O}^6\text{-meG}$  in DNA and for alkylguanine alkyltransferase (AGT) were as described previously (Souliotis and Kyrtopoulos, 1989; Souliotis *et al.*, 1990).

For the time course of change in level of DNA adducts in blood cells, similar results were obtained by averaging values from all the monkeys (Series I and II) and by comparing different experimental treatments of the same monkey (Series I). Therefore only the former data are shown (Fig. 2).

#### RESULTS

DNA alkylation data were obtained for 32 types of monkey tissues. The repair enzyme AGT was measured in many of these tissues in a separate monkey, and the results are given in the figure legends and Table I. The AGT values are in good agreement with those reported for comparable *Macaca cynomolgus* monkey and human tissues (Hall *et al.*, 1985; Loktionova *et al.*, 1993). Amounts of AGT varied over a 30-fold range in the monkey tissues.

##### Gastrointestinal tract

Striking findings were obtained (Fig. 1a). First, after administration of NDMA only, adduct levels in stomach mucosa were similar to, indeed consistently slightly higher than, those in liver; these were the highest levels of all tissues, in each of the 5 monkeys tested.  $\text{O}^6\text{-meG}$  levels in mucosa of esophagus, pancreas, large bowel and small bowel ( $1.0 \pm 0.2 \mu\text{mol/mol G}$ ), were also within 50% of those in liver. It is evident that, in apparent contrast to rodents, these important cancer targets share with liver an equivalent likelihood of sustaining DNA damage after NDMA exposure.

TABLE I. – DNA ADDUCTS AND AGT IN OTHER TISSUES<sup>1</sup>

Tissue	$\text{O}^6\text{-meG}$ ( $\mu\text{mol/mol G}$ )		AGT (fmol/ $\mu\text{g}$ DNA)
	NDMA	Ethanol/NDMA	
Bone marrow	$0.5 \pm 0.2$	$1.3 \pm 0.2$	7.10
Spleen	$0.4 \pm 0.2$	$5.3 \pm 0.5$	16.30
Lymph nodes	$0.1 \pm 0.04$	NT	5.14
Prostate	$0.3 \pm 0.1$	NT	NT
Adrenal	$0.3 \pm 0.1$	$1.5 \pm 0.3$	3.92
Pituitary	$0.2 \pm 0.1$	NT	NT
Salivary gland	$0.2 \pm 0.02$	NT	4.55
Skeletal muscle	$0.2 \pm 0.1$	$0.8 \pm 0.2$	2.38
Heart muscle	$0.4 \pm 0.1$	$2.1 \pm 0.5$	2.97
Heart endothelium	$0.3 \pm 0.1$	$1.9 \pm 0.5$	NT
Skin	$0.3 \pm 0.1$	$0.8 \pm 0.2$	4.80
Lung, peripheral	$0.1 \pm 0.03$	$0.5 \pm 0.1$	14.64
Lung, bronchus	$0.08 \pm 0.01$	$0.4 \pm 0.1$	10.13

<sup>1</sup>NT, not tested.

Secondly, co-exposure to ethanol resulted in marked increases in the level of  $\text{O}^6\text{-meG}$  adduct in esophageal mucosa (17-fold), colon mucosa (11.9-fold) and pancreas (6.4-fold). A much smaller effect was seen in stomach (2.1-fold). There was no effect in liver, which was the only tissue for which no enhancement by ethanol was seen.

Greatest amounts of AGT were present in liver and stomach and 50% less in pancreas and large and small intestines. Esophageal AGT was about 25% that in stomach.

##### Urogenital tract

After NDMA only, urinary bladder, ovary and uterus presented amounts of DNA adducts that were again within 50% of those in liver (Fig. 1b). Levels were lower in the kidneys and were greater in the right kidney in 4 of the 5 monkeys ( $p = 0.018$ , paired two-tailed  $t$  test). Interestingly, the right kidney had about 30% less AGT activity. Amounts of adduct in right and left testes were similar,  $0.5 \pm 0.1 \mu\text{mol/mol G}$ .

Ethanol co-exposure greatly increased the amounts of DNA adducts in all of these tissues, 10.9-fold for urinary bladder, 4- to 5-fold for kidney, 7.8-fold for uterus and 9.4-fold for ovary.

Kidney, ovary and uterus had intermediate levels of AGT, about 30–40% that of liver, whereas bladder AGT was 10–12% of liver.

##### Nasal cavity mucosa and brain

Adduct levels were similar in all these tissues after NDMA only and were about 20% those in liver (Fig. 1c). Oral mucosa adduct levels were similar,  $0.3 \pm 0.1 \mu\text{mol/mol G}$ . Ethanol treatment resulted in a 5.1-fold increase in adducts of nasal cavity mucosa DNA, 7- to 8-fold increases in cerebrum and cerebellum and a 10-fold elevation in the brain stem. AGT values in the 3 areas of brain were low, 3–8% of liver.

##### White blood cells and other tissues

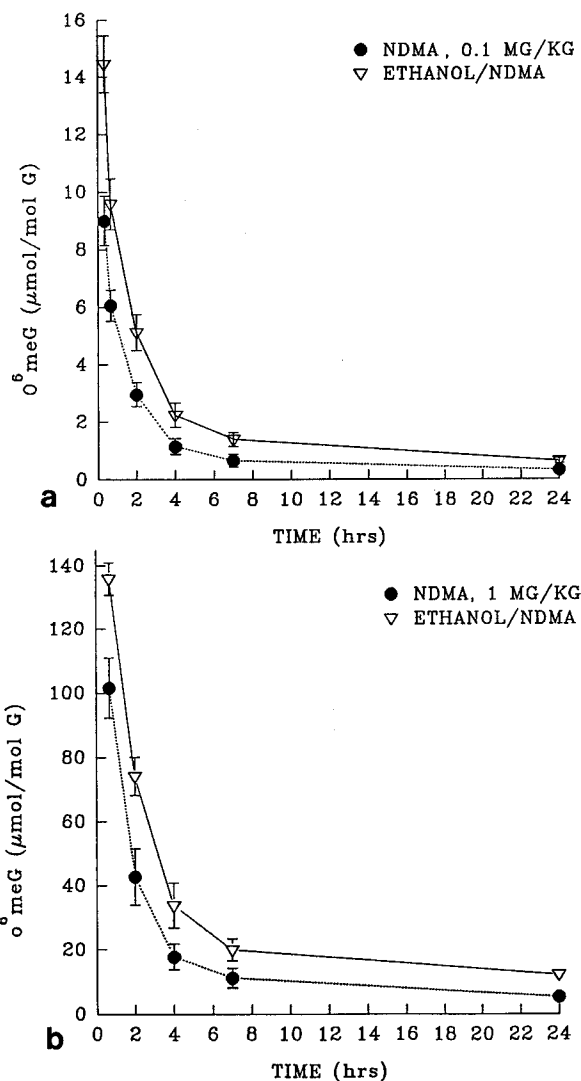
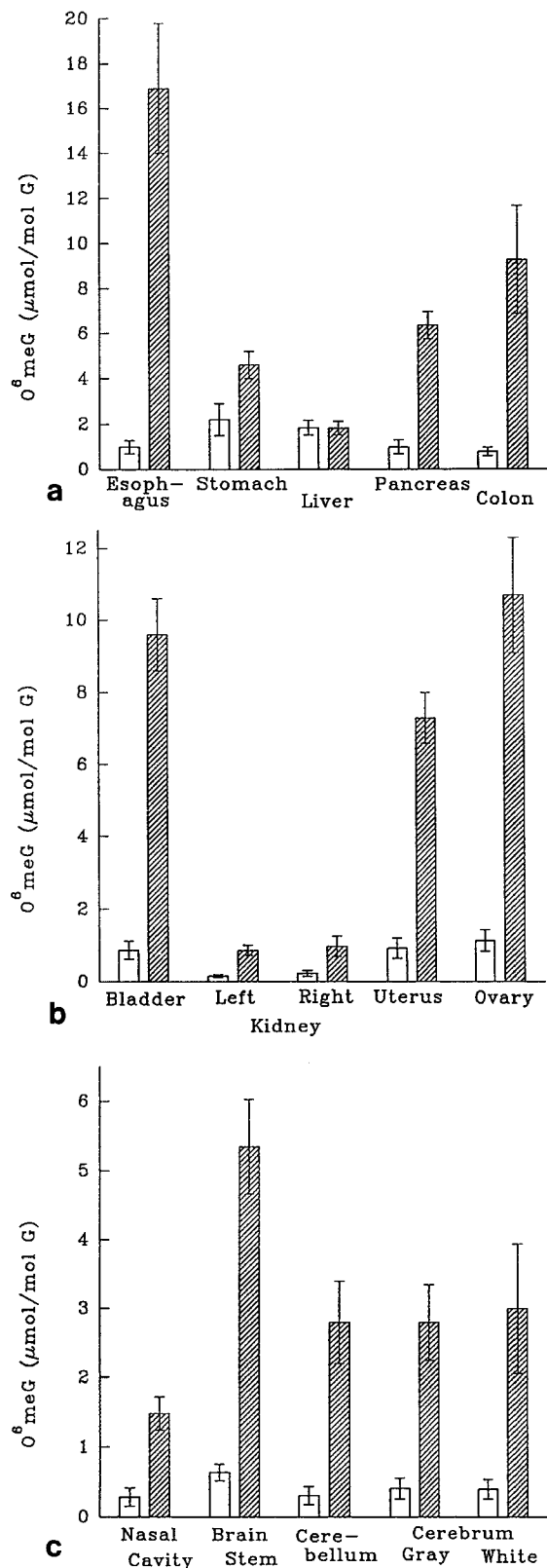
Sequential sampling of blood at intervals after dosing with 0.1 mg/kg NDMA showed that adduct levels in white blood cells were already maximum at 20 min and decreased thereafter, with apparent biphasic repair (Fig. 2a). In the presence of ethanol, adduct levels were 1.5- to 2-fold greater throughout this course. With the higher dose of 1 mg/kg, adduct levels were more than 10-fold greater than with 0.1 mg/kg (Fig. 2b). The ethanol effect and the adduct loss curves were similar to those obtained with the lower NDMA dose.

Data for other tissues tested are presented in Table I. These had adduct levels that were 10–20% of those in liver and showed in general a 3- to 6-fold effect of ethanol, except for spleen, where a 12.6-fold increase was noted. AGT levels were moderate in spleen and lung, and low in the other tissues tested.

## DISCUSSION

The results of our study with a non-human primate model provide confirmation of 2 hypotheses regarding a possible role for nitrosamine in human carcinogenesis. The first hypothesis

is that nitrosamines present in food, beverages or tobacco-related products, or those formed *in vivo* by spontaneous or microbe-catalyzed nitrosation, could initiate cancers in a spectrum of human tissues. We have now shown that, after oral administration of a low dose of NDMA to monkeys, a promutagenic DNA adduct was present, at levels similar to those in liver, in many of these target tissues: esophagus, stomach, pancreas, large bowel, urinary bladder, uterus, ovary and white blood cells. Indeed, amounts of adducts in stomach mucosa were consistently higher than in liver. Therefore,



**FIGURE 2** –  $\text{O}^6\text{-meG}$  in DNA of blood cells taken at the indicated intervals after NDMA only (dotted line, closed circles) or ethanol/NDMA (solid line, open triangles). (a) NDMA at 0.1 mg/kg. (b) NDMA at 1.0 mg/kg. Values are the average of 2–5 samples  $\pm$  SD.

**FIGURE 1** –  $\text{O}^6\text{-meG}$  in DNA, average  $\mu\text{mol/mol G} \pm$  SD. Open bars, NDMA only; hatched bars, ethanol/NDMA. (a) Gastrointestinal tissues. The levels of AGT as fmol/ $\mu\text{g}$  DNA, measured in a separate female monkey, were: esophagus, 9.35; stomach, 37.84; liver, 47.42; pancreas, 20.14; colon, 19.87; small intestine, 18.90. (b) Urogenital tissues. AGT values were: bladder, 5.63; kidney, left, 19.45; kidney, right, 14.26; uterus, 4.34; ovary, 12.66. (c) Nasal cavity and brain. AGT values were: brain stem, 3.64; cerebrum, gray, 1.5; cerebrum, white, 2.31.



substantial DNA damage is sustained in these tissues, and tumors could be initiated.

The second hypothesis is that alcohol use increases the risk of certain cancers, at least in part by inhibiting hepatic clearance and thereby causing increased internal exposure. Our monkey study affirms this hypothesis, showing that co-exposure to ethanol resulted in large increases in DNA adducts in certain tissues. The change in esophagus was particularly striking, 17-fold, and matches closely the 18-fold increase in risk of human esophageal cancer that occurs with tobacco plus heavy alcohol use (Tuyns *et al.*, 1980). While this quantitative similarity may be coincidental, it does indicate that the suppression-of-clearance effect *could* by itself account for the elevation in risk. A number of other monkey tissues showed an 8-fold or greater change: cerebellum, brain stem, large bowel, spleen, urinary bladder, ovary and uterus.

Only the liver showed no enhancement of adducts after ethanol co-treatment. Similar findings have been reported for rats (Swann *et al.*, 1984) and mice (Anderson, 1992). Thus, this action of ethanol would not contribute to increased risk for liver cancer. Why the liver differs from the other tissues in this regard is unknown. Possibly, almost all of the activation and catabolism of NDMA in the liver is catalyzed by P450 2E1, which is, obviously, strongly inhibited by ethanol, so that increased exposure is exactly counterbalanced by decreased activation rate. In other tissues, one or more other P450s, or other enzymes, that are less sensitive to ethanol inhibition, may activate NDMA.

The various tissues clearly differed in both amount of DNA adduct present 4 hr after treatment with NDMA alone, and in relative effect of ethanol, and so were each actively influencing the outcome of exposure. In general, tissues with high adducts after NDMA only also showed a marked ethanol effect: esophagus, large bowel, urinary bladder, ovary and uterus. Apparent exceptions were the white blood cells, with a relatively high adduct level but a small ethanol effect, and spleen and cerebellum, characterized by relatively low adducts

but a large ethanol effect. Amounts of NDMA activating and DNA repair enzymes, and possible suicide-destructive effects of NDMA, as well as inhibitory effects of ethanol on these enzymes, might be expected to interplay uniquely in each tissue.

The relatively high level of adducts in blood cells is of interest, since these cells are used in human biomarker studies. The monkey data suggests that the blood cell values may predict rather closely the adduct levels in sensitive target organs. It was puzzling that adduct formation in the blood cells apparently reached a peak shortly after NDMA dosing and then rapidly declined even in the presence of ethanol. NDMA, at least after the 1 mg/kg dose, would have continued to circulate at maximum blood levels for more than 6 hr, until the suppression of hepatic clearance was relieved by elimination of the ethanol. It is possible that an ethanol-insensitive enzyme, with a relatively high  $K_m$  for NDMA, in intestinal mucosa or periportal cells of liver, was responsible for the initial activation leading to DNA adducts in the white blood cells. Further studies involving i.v. administration are planned to test this possibility.

The levels of the AGT repair enzyme tended, as might be expected, to be greatest in tissues that would interact directly with environmental chemicals: mucosa of the gastrointestinal tract, liver, kidneys and lung. The prominence of AGT in gastric mucosa, second in amount only to liver, further attests to the likelihood of alkylation damage in this tissue. The presence of high levels of O<sup>6</sup>-meG in gastric mucosa DNA 4 hr after treatment, in spite of abundant AGT, suggests that initial damage could have been much higher.

In conclusion, our monkey data support the hypotheses that nitrosamines contribute to cancer risk of the gastrointestinal and urogenital tracts, and that ethanol enhances risks of some of these cancers by suppressing first-pass hepatic clearance of nitrosamines. Further work is needed, particularly on the mechanisms of NDMA activation in tissues other than liver, which are important human cancer targets.

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